EXHIBIT G

Mutations that suppress the thermosensitivity of green fluorescent protein

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Background: The green fluorescent protein (GFP) of the jellyfish *Aequores victoria* has recently attracted great interest as the first example of a cloned reporter protein that is intrinsically fluorescent. Although successful in some organisms, heterologous expression of GFP has not always been straight forward. In particular, expression of GFP in cells that require incubation temperatures around 37 °C has been problematic.

Results: We have carried out a screen for mutant forms of GFP that fluoresce more intensely than the wild-type protein when expressed in *E. coli* at 37 °C. We have characterized a bright mutant (GFPA) with reduced sensitivity to temperature in both bacteria and yeast, and have shown that the amino acids substituted in GFPA act by preventing temperature-dependent misfolding of the GFP apoprotein. We have shown that the excitation and emission spectra of GFPA can be manipulated by site-directed mutagenesis without disturbing its improved folding characteristics, and have produced a thermostable folding mutant (GFP5) that can be efficiently excited using either long-wavelength ultraviolet or blue light. Expression of GFP5 results in greatly improved levels of fluorescence in both microbial and mammalian cells cultured at 37 °C.

Conclusions: The thermotolerant mutants of GFP greatly improve the sensitivity of the protein as a visible reporter molecule in bacterial, yeast and mammalian cells. The fluorescence spectra of these mutants can be manipulated by further mutagenesis without deleteriously affecting their improved folding characteristics, so it may be possible to engineer a range of spectral variants with improved tolerance to temperature. Such a range of sensitive reporter proteins will greatly improve the prospects for GFP-based applications in cells that require relatively high incubation temperatures.

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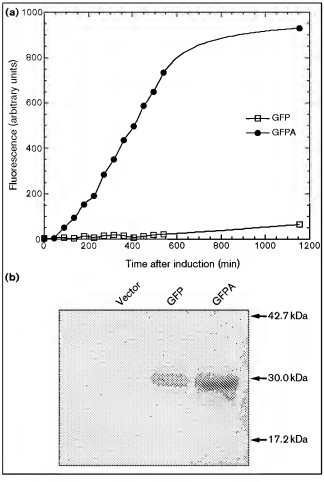
Background

The green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequora victoria* emits green light (λ_{max} = 509 nm) upon excitation with long-wavelength ultraviolet (UV) or blue light (maximally at 400 nm, with a secondary peak at 475 nm) [1]. GFP is unusual amongst photoproteins and fluorescent proteins in that its chromophore is intrinsic rather than extrinsic: the tripeptide Ser65–Tyr66–Gly67 of the GFP primary sequence undergoes cyclization and oxidation to form a p-hydroxybenzylideneimidazolidinone chromophore [2–4]. This process is believed either to be autocatalytic or to require only ubiquitous cellular factors, because the *gfp* cDNA has been cloned [5] and fluorescence observed upon expression in a diverse range of organisms (see [4] for review). This characteristic makes GFP extremely attractive as an alternative to current reporter molecules, because it allows the direct visualization of gene expression and subcellular localization of fusion proteins in living cells, without the need for invasive techniques or the addition of cofactors [6,7].

The fluorescence spectra of GFP are generally well suited for its application as a fluorescent reporter molecule. The 400 nm excitation peak is useful for direct visual screening of whole organisms or colonies of cells with a long-wavelength UV lamp. The 475 nm excitation peak is important for applications such as fluorescence microscopy, confocal laser scanning microscopy and fluorescence-activated cell sorting, where filter sets optimized for the detection of fluorescein derivatives or blue laser excitation are commonly used. The list of potential applications of GFP has been further extended by the isolation of mutants with altered excitation and emission spectra [3,8–10] which open the way for simultaneous monitoring of multiple cellular events [11] or analysis of protein–protein interactions by fluorescence resonance energy transfer [12].

Although GFP has many obvious advantages as a fluorescent reporter molecule, expression of the protein in heterologous systems is not necessarily straight forward [4]. For example, safe expression of high levels of GFP in transgenic *Arabidopsis thaliana* has required the elimination

Figure 1



Induction of GFP and GFPA expression in *E. coli*. **(a)** Fluorescence of cells expressing GFP or GFPA. *E. coli* XL1Blue cells containing pSE-GFP or pSE-GFPA were grown at 37 °C to an optical density of 0.2 at 600 nm and then induced with 0.5 mM IPTG. At the times shown, samples were withdrawn, diluted to an optical density of 0.2 at 600 nm, and the fluorescence (λ_{ex} = 397 nm, λ_{em} = 508 nm) measured at 25 °C. **(b)** Accumulation of recombinant protein in cells expressing GFP or GFPA. Total protein prepared from cells collected 270 min after induction with IPTG was western blotted (with anti-GFP antibodies) as described in Materials and methods. Protein was from cells carrying the pSE380 vector (lane marked Vector), pSE-GFP (GFP) and pSE-GFPA (GFPA).

of a cryptic intron from the coding sequence and compartmentalization of the protein to the endoplasmic reticulum ([13]; J.H., K.R.S., D. Prasher and S. Hodge, unpublished observations). Also, expression of GFP in mammalian cells has been described as highly variable [14], often requiring a strong promoter and decreased incubation temperature for good results [15–17]. Other researchers have found that development of fluorescence is similarly favoured by a lower incubation temperature during expression of GFP in bacteria [18] and yeast [19]. These observations suggest that expression of GFP in cells that require higher incubation temperatures may be far from optimal.

In this study, we have isolated a mutant of GFP (GFPA) that fluoresces 35-fold more intensely than wild-type GFP when expressed in *Escherichia coli* at 37 °C. We demonstrate that this increased fluorescence results primarily from suppression of a temperature-dependent defect in the folding of the GFP apoprotein. We also describe manipulation of the fluorescence spectra of GFPA by site-directed mutagenesis to produce a thermostable folding mutant, GFP5, which can be efficiently excited using either long-wave UV or blue light, and expression of which results in significantly improved levels of fluorescence in mammalian cells cultured at 37 °C. These variants will be of particular benefit for the application of GFP in experimental systems that require incubation temperatures around 37 °C.

Results

Isolation and characterization of a GFP mutant with enhanced fluorescence at 37 °C

We have altered the codon usage of the jellyfish cDNA coding for GFP in order to eliminate a cryptic intron that is efficiently recognized during expression in *A. thaliana* ([13]; J.H., K.R.S., D. Prasher and S. Hodge, unpublished observations). The modified gene, *mgfp4*, was subjected to random mutagenesis using the polymerase chain reaction (PCR), and a library of mutant genes was constructed in the plasmid Bluescript II KS (+). The mutant library was introduced into *E. coli* and expressed overnight at 37 °C. Approximately 10 000 colonies on agar plates were illuminated with long-wavelength UV light and visually screened for increased fluorescence. Two of the brightest clones (designated GFPA and GFPB) obtained from this screen were chosen for further analysis.

In order to localize the positions of the mutations responsible for the bright phenotypes of GFPA and GFPB, various restriction fragments of the mutant genes (*mgfpA* and *mgfpB*, respectively) were recombined with the wild-type *mgfp4* gene (see Materials and methods for details). In each case, the mutation(s) responsible for the bright phenotype were found to lie within a 336 base-pair *ClaI*–*SacI* fragment at the 3′ end of the gene. Sequencing of the *ClaI*–*SacI* fragment of *mgfpB* revealed the presence of a single coding alteration, Val163→Ala (V163A). This same change was found in combination with a second coding alteration, Ser175→Gly (S175G), in the *ClaI*–*SacI* fragment of *mgfpA*. The S175G change contributed to the phenotype of GFPA: cells expressing the GFPA protein were clearly more fluorescent than those expressing GFPB, so the GFPA mutant was selected for further characterization.

In order to assess quantitatively the difference in fluorescence between strains expressing GFP and GFPA, we cloned *mgfp4* and *mgfpA* downstream of the inducible *trc* promoter of the expression vector pSE380. The fluorescence of similar numbers (equal optical densities) of *E. coli* cells containing the resulting plasmids was then measured

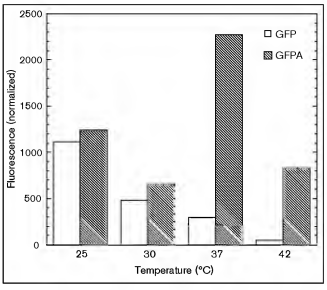
Table 1

Fluorescence and accumulation of recombinant protein in bacteria expressing GFP or GFPA at different temperatures.

Temperature (°C)	Fluorescence (arbitrary units)*		Relative amount of intracellular recombinant protein†	
	GFP	GFPA	GFP	GFPA
25	328.4	722.2	0.29	0.58
30	100.5	541.1	0.21	0.82
37	67.9	2273.0	0.23	1.00
42	9.2	369.4	0.17	0.44

*XL1-Blue cells containing pSE-GFP or pSE-GFPA were grown to an optical density of 0.2 at 600 nm and then induced overnight with 0.2 mM IPTG. The fluorescence of cell samples was measured as in Fig. 1a.
†Total protein prepared from the cell samples used for fluorescence measurements was western blotted as in Fig. 1b and the amounts of intracellular recombinant protein determined by quantification of band intensities. Data have been normalized to the highest value that was obtained (7.8×10^8 arbitrary units for GFPA expressed at 37 °C).

Figure 2



Relative proportions of GFP and GFPA that are fluorescent in bacterial cells grown at different temperatures. Values were calculated by normalization of fluorescence values against the amount of intracellular recombinant protein for cultures grown at 25, 30, 37 or 42 °C (Table 1).

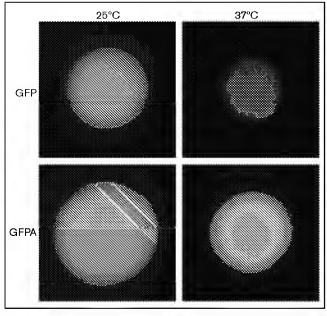
at various times following induction of protein synthesis at 37 °C with isopropyl β-D-thiogalactoside (IPTG; see Fig. 1a). At 4.5 hours after induction, cells expressing GFPA were observed to fluoresce 20-fold more intensely than those expressing GFP, a figure which increased to 35-fold by the time the cells had entered stationary phase (9 hours after induction). To determine whether the enhanced fluorescence of cells expressing GFPA was due to increased levels of protein expression, total protein was prepared from cells sampled at the 4.5 hour time point and the amount of intracellular GFP estimated by western immunoblot. As can be seen in Figure 1b, GFPA accumulates inside cells to a significantly higher level than does GFP. However, the difference in protein levels as estimated by quantification of band intensities is 2.4-fold, not nearly enough to account for the 20-fold difference in fluorescence levels observed at this time point. This result suggests either that a large proportion of GFP that is expressed in cells at 37 °C is non-fluorescent and that the substitutions present in GFPA enhance the formation or stability of the mature fluorescent form, or that the substitutions in GFPA substantially increase the intrinsic fluorescence of the mature protein. Comparing the growth curves of strains expressing GFP or GFPA with the growth curve of a non-expressing strain (data not shown) indicated that expression of these proteins has little adverse effect on the growth of bacterial cells.

The amino-acid substitutions present in GFPA enhance maturation at elevated temperatures in bacteria and yeast

Experiments with a GFP–nucleoplasmin fusion protein have indicated that maturation of GFP to the fluorescent form may be sensitive to temperature during expression in the yeast *Saccharomyces cerevisiae* [19]. To test whether the same could be true of expression in *E. coli*, and whether the substitutions present in GFPA enhance maturation by suppressing any such sensitivity, we examined expression of GFP and GFPA over a range of temperatures. Strains expressing GFP or GFPA were grown overnight at temperatures between 25 °C and 42 °C. For each culture, the fluorescence of an equal optical density of cells was measured at 25 °C and the amount of intracellular recombinant protein determined by western blot (Table 1). Fluorescence values were then normalized against the amount of recombinant protein present in the cells to give a relative measure of the proportion of intracellular GFP that is fluorescent for each culture. The results (Fig. 2) clearly show that the proportion of GFP in the cells that is fluorescent steadily decreases with increasing incubation temperature, indicating that either mature GFP or the pathway leading to its formation is temperature-sensitive.

In order to test whether mature GFP itself is thermosensitive, we examined the effect of temperature on its fluorescence in bacterial cells. *E. coli* cells expressing GFP were grown to mid-log phase at 25 °C and treated with 50 μg ml^{-1} chloramphenicol to inhibit further protein synthesis. The culture was then divided in two and one half incubated at 25 °C and the other at 42 °C for an additional 4.5 hours. Comparison of the fluorescence intensities of the two samples revealed no significant difference (data not shown), indicating that the fluorescence of mature GFP synthesized prior to the chloramphenicol block is unaffected by temperature. This finding is consistent with the fact that mature GFP is a highly stable molecule whose fluorescence *in vitro* is unaffected by temperatures up to

Figure 3



The substitutions present in GFPA cure the thermosensitivity of GFP expression in yeast. Strains of *S. cerevisiae* MGLD-4a expressing either GFP or GFPA were incubated at 25 °C and 37 °C on synthetic drop-out agar lacking uracil [33]. Colonies (each approximately 1.5 mm in diameter) were visualized as described in Materials and methods using the Leitz-D filter set suitable for UV excitation of GFP.

65 °C [20], and with the results of a similar experiment that was carried out in yeast cells using a GFP–nucleoplasmin fusion protein [19]. We therefore conclude that higher incubation temperatures interfere with the post-translational maturation of GFP rather than causing inactivation of the mature protein. Moreover, the primary effect of the substitutions present in GFPA is to enhance the proportion of GFP that is fluorescent at higher temperatures, rather than simply enhancing the intrinsic fluorescence properties of mature GFP.

To determine whether the substitutions present in GFPA were also capable of suppressing thermosensitivity of GFP maturation in yeast, strains of *S. cerevisiae* expressing either GFP or GFPA were grown on agar plates at either 25 °C or 37 °C. The results, shown in Figure 3, confirm that expression of GFP is temperature-sensitive in yeast and demonstrate that this phenomenon is also suppressed by the substitutions present in GFPA. These results indicate that the thermosensitivity of GFP maturation may be a common phenomenon that can be suppressed by the amino-acid substitutions present in GFPA.

Chromophore oxidation during maturation of GFP and GFPA

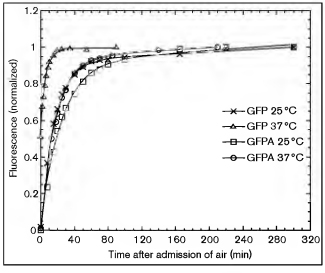
The post-translational maturation of GFP to the fluorescent form involves a number of steps [2–4]. First, the GFP apoprotein must presumably fold into a catalytically active conformation that facilitates cyclization and oxidation of the tripeptide Ser65–Tyr66–Gly67. The mature protein must then be correctly folded to maintain its fluorescent properties, presumably to protect the chromophore from solvent effects [21–23]. In principle, any of these steps could be sensitive to temperature and could thus be responsible for the observed thermosensitivity of GFP during maturation.

Because the oxidation reaction involved in chromophore formation appears to require molecular oxygen, Heim *et al.* [10] were able to measure the reaction rate by expressing GFP in *E. coli* under anaerobic conditions and then monitoring the development of fluorescence after admission of air. To determine whether this reaction is temperature-sensitive and whether the substitutions present in GFPA enhance its rate at higher temperatures, we measured the rates of oxidation of GFP and GFPA at both 25 °C and 37 °C. For our experiments, we chose to use a yeast expression system which provided for better growth and expression levels under anaerobic conditions than bacteria. *S. cerevisiae* cells expressing either GFP or GFPA were grown under anaerobic conditions overnight at 30 °C. As was the case in bacteria, *S. cerevisiae* cells exhibited no fluorescence following anaerobic growth. Air was then admitted to the cultures and the development of fluorescence followed at either 25 °C or 37 °C. As reported previously [10], each oxidation proceeded as a pseudo-first-order reaction (Fig. 4). The time constant measured for the oxidation of GFP at 37 °C (5.9 ± 0.1 min) was found to be approximately 3-fold faster than that measured at 25 °C (16.2 ± 0.3 min), indicating that the post-translational oxidation of the GFP chromophore is not the step responsible for the temperature sensitivity of maturation. In confirmation of this conclusion, the time constants derived for GFPA at both 25 °C and 37 °C (22.5 ± 1.4 min and 18.1 ± 0.4 min, respectively) were actually slower than those measured for GFP.

Apo-GFP folds improperly at elevated temperatures

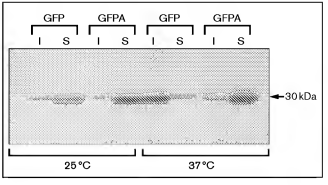
The improper folding of proteins often results in their aggregation into insoluble inclusion bodies during expression in *E. coli* [24]. To determine whether the proper folding of GFP might be temperature-sensitive and whether the substitutions present in GFPA act by enhancing proper folding at increased temperatures, we examined the solubilities of the two proteins during expression in *E. coli* at 25 °C and 37 °C. Bacterial cells expressing GFP or GFPA were grown overnight at either temperature, lysed, and the soluble and insoluble fractions separated by centrifugation (see Materials and methods for details). In each case, fluorescence was found almost exclusively in the soluble fraction. The amount of GFP or GFPA present in each fraction was then estimated by western blot (Fig. 5). At 25 °C, both GFP and GFPA were found predominantly in the soluble fraction, indicating relatively efficient folding of both proteins. At 37 °C, however, the majority of GFP was found as non-fluorescent protein in the insoluble

Figure 4



Rates of chromophore oxidation of GFP and GFPA at 25 °C and 37 °C. Rates of oxidation were measured as described in Materials and methods. Curves were fitted to single first-order kinetics and time constants calculated using the KaleidaGraph 3.0 program (Abelbeck Software). All curves have been normalized to an asymptotic value of 1.0.

Figure 5



Solubilities of GFP and GFPA during expression in bacterial cells at 25 °C and 37 °C. Soluble and insoluble fractions of *E. coli* cells expressing GFP or GFPA at 25 °C or 37 °C were prepared as described in Materials and methods. The relative amount of GFP or GFPA present in 2.5 µl of each fraction was determined by western blot (with anti-GFP antibodies). I, insoluble fraction; S, soluble fraction.

fraction, whereas most GFPA was still soluble. This result indicates that the temperature-sensitivity of GFP maturation is due primarily to improper folding at higher temperatures, and that this defect is suppressed by the amino-acid substitutions present in GFPA.

In order to obtain information on which species in the maturation pathway of GFP misfolds at higher temperatures, we examined the absorption spectrum of denatured protein isolated from inclusion bodies. If GFP undergoes the cyclization reaction prior to aggregation, protein from inclusion bodies should show an absorption in the near UV/blue region characteristic of the GFP chromophore in either the mature or reduced state [25,26]. On the other hand, if unmodified GFP (apo-GFP) is the aggregating species, no such absorption should be observable in this region. To assist in the purification of protein for absorption measurements, we used polyhistidine-tagged GFP that was expressed from the *tr* promoter of pSE380. Polyhistidine-tagging of GFP did not detectably affect the temperature-sensitivity of its maturation (data not shown).

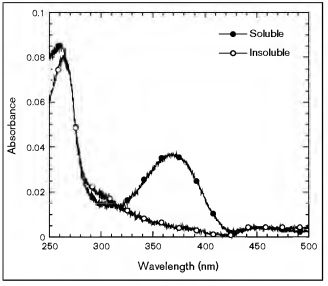
Polyhistidine-tagged GFP was isolated from the inclusion bodies of bacterial cells grown at 37 °C and, as a positive control, from the soluble fraction of cells grown at 25 °C. Equal concentrations of protein from the two preparations were prepared in resolubilization buffer, denatured by treatment with heat, and their absorption spectra recorded. As can be seen in Figure 6, denatured protein derived from the soluble fraction of cells grown at 25 °C showed a characteristic absorption peak similar to that of acid-denatured GFP [25]. By contrast, protein purified from inclusion bodies of cells grown at 37 °C showed no such absorption, indicating that the aggregating species had not formed a chromophore. Taken together, these results indicate that the temperature-sensitivity of GFP maturation is due primarily to the failure of the unmodified apoprotein to fold into its catalytically active conformation at higher temperatures. Furthermore, the amino-acid substitutions present in GFPA suppress this defect by enhancing proper folding at elevated temperatures.

Modification of the fluorescence spectra of GFPA

Fluorescence spectroscopy of purified polyhistidine-tagged GFP and GFPA revealed that the fluorescence spectra of GFPA are essentially unchanged from those of GFP except for a small shift in the relative amplitudes of the 400 nm and 475 nm excitation peaks (Fig. 7). Although this spectral shift might be advantageous for applications that use 400 nm excitation, the reduced relative amplitude of the 475 nm peak is detrimental for those that use blue-light excitation. For many purposes, the ideal spectral variant would be a protein which could be efficiently excited at either of these wavelengths, and so could be used for a range of applications.

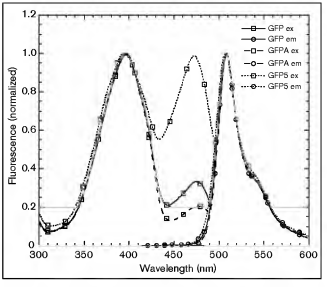
Recently, it has been demonstrated that, as we have observed here, the relative amplitudes of the excitation peaks of GFP can be altered by mutagenesis [3,8–10]. A number of these mutations, like the substitutions present in GFPA, are located in the carboxy-terminal region of the protein. It has been hypothesized that these mutations affect the microenvironment of the chromophore so as to influence the equilibrium between the two spectroscopic states of the chromophore that give rise to the two excitation peaks [3,9]. Indeed, a number of these susbtituted

Figure 6



Absorption spectra of GFP isolated from the soluble fraction of cells grown at 25 °C (soluble) and from inclusion bodies of cells grown at 37 °C (insoluble). Protein purifications were carried out as described in Materials and methods. Samples, each containing 10 μM GFP in resolubilization buffer, were denatured by heating at 75 °C for 5 min prior to spectroscopy.

Figure 7



Excitation (ex) and emission (em) spectra of GFP, GFPA and GFP5. Protein concentrations were 23.5 μg ml^{-1} in PBS (pH 7.4). All spectra have been normalized to a maximum value of 1.0.

residues have subsequently been found to lie close to the chromophore in the three-dimensional structure [22,23]. One mutation, I167T, has been shown to increase the amplitude of the 475 nm excitation peak relative to that of the 400 nm peak [3]. In order to test whether the combination of the I167T substitution with the substitutions present in GFPA might increase the amplitude of the 475 nm peak relative to the 400 nm peak, the sequence of *mgfp4* was modified so as to code for the V163A, S175G and I167T substitutions. Although fluorescence was readily observable using either UV- or blue-light excitation when the resulting gene was expressed in bacterial cells, very little fluorescence was observed when it was expressed in transgenic *A. thaliana* plants (S. Hodge, K.R.S. and J.H., unpublished observations).

A number of additional changes to codon usage were therefore made, in order to eliminate any potential plant intron recognition sequences, and these have been shown to restore fluorescence upon expression of the modified

Figure 8

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GTC ACT ACT TTC TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAA CGG    gfp
GTC ACT ACT TTC TCT TAT GGT GTT CAA TGC TTT TCA AGA TAC CCA GAT CAT ATG AAG CGG    mgfp5
 V  T  T  F  S  Y  G  V  Q  C75  F  S  R  Y  P  D  H  M  K  R80

CAT GAC TTT TTC AAG AGT GCC ATG CCC GAA GGT TAT GTA CAG GAA AGA ACT ATA TTT TTC    gfp
CAC GAC TTC TTC AAG AGC GCC ATG CCT GAG GGA TAC GTG CAG GAG AGG ACC ATC TTC TTC    mgfp5
 H  D  F  F  K  S  A  M  P  E95  G  Y  V  Q  E  R  T  I  F  F100

AAA GAT GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT GAA GGT GAT ACC CTT GTT    gfp
AAG GAC GAC GGG AAC TAC AAG ACA CGT GCT GAA GTC AAG TTT GAG GGA GAC ACC CTC GTC    mgfp5
 K  D  D  G  N  Y  K  T  R  A115 E  V  K  F  E  G  D  T  L  V120

AAT AGA ATC GAG TTA AAA GGT ATT GAT TTT AAA GAA GAT GGA AAC ATT CTT GGA CAC AAA    gfp
AAC AGG ATC GAG CTT AAG GGA ATC GAT TTC AAG GAG GAC GGA AAC ATC CTC GGC CAC AAG    mgfp5
 N  R  I  E  L  K  G  I  D  F135 K  E  D  G  N  I  L  G  H  K140

TTG GAA TAC AAC TAT AAC TCA CAC AAT GTA TAC ATC ATG GCA GAC AAA CAA AAG AAT GGA    gfp
TTG GAA TAC AAC TAC AAC TCC CAC AAC GTA TAC ATC ATG GCC GAC AAG CAA AAG AAC GGC    mgfp5
 L  E  Y  N  Y  N  S  H  N  V155 Y  I  M  A  D  K  Q  K  N  G160

                   I                                    S
ATC AAA GTT AAC TTC AAA ATT AGA CAC AAC ATT GAA GAT GGA AGC GTT CAA CTA GCA GAC    gfp
ATC AAA GCC AAC TTC AAG ACC CIC CAC AAC ATC GAA GAC GGC AGC GTG CAA CTC GCT GAT    mgfp5
 I  K  A  N  F  K  T  R  H  N170 I  E  D  G  S  V  Q  L  A  D180
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Partial sequences of *gfp* and *mgfp5*. The sequence of *gfp* is as described for the *gfp10* cDNA [5], except that codon 80 contains a change from CAG to CGG resulting in replacement of a glutamine with arginine, as noted by Chalfie *et al.* [6]. The cryptic plant intron present in *gfp* ([13]; J.H., K.R.S., D. Prasher and S. Hodge, unpublished observations) is underlined. The nucleotide sequence alterations present in *mgfp5* are shown in bold. All alterations are non-coding except for where the amino acids of GFP that were substituted are shown in bold above the nucleotide sequence. The tripeptide involved in chromophore formation is boxed.

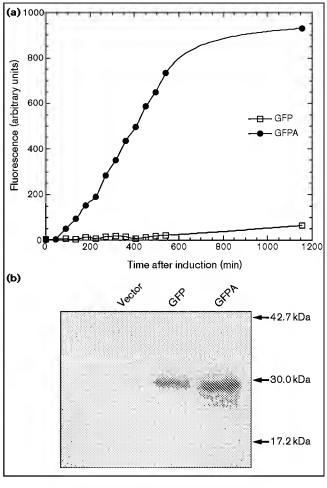
gene, *mgfp5*, in *A. thaliana* (J.H., K.R.S., D. Prasher and S. Hodge, unpublished observations). The sequence differences between *mgfp5* and the original *gfp* gene are summarized in Figure 8. To examine the spectral properties of GFP5 in more detail, polyhistidine-tagged protein was purified and its excitation and emission spectra analysed by fluorescence spectroscopy. As can be seen in Figure 7, GFP5 has two excitation peaks (maxima at 395 nm and 473 nm) of almost exactly equal amplitude and an emission spectrum (λ_{max} = 507 nm) largely unchanged from that of GFP. To determine whether GFP5 has retained the thermotolerant phenotype of GFPA, the fluorescence of bacterial cells expressing GFP5 was compared to that of cells expressing GFP at 37 °C. Cells were induced with IPTG for 5 hours at 37 °C and the fluorescence (λ_{ex} = 395 nm or 473 nm, λ_{em} = 507 nm) of equal optical densities of cells measured. Cells expressing GFP5 were found to fluoresce 39-fold more intensely than cells expressing GFP when excited at 395 nm, and 111-fold more intensely when excited at 473 nm, indicating that GFP5 has retained the thermotolerant phenotype of GFPA.

These results demonstrate that it is possible to manipulate the fluorescence spectra of GFPA by introducing additional substitutions into the protein without deleteriously affecting its improved folding characteristics. To explore this possibility further, we introduced a mutation coding for the Y66H chromophore substitution into the *mgfp4* and *mgfpA* genes. The Y66H substitution [3] dramatically blue-shifts both the excitation and emission spectra of GFP to give a 'blue fluorescent protein'. GFPA containing the Y66H substitution, GFPA(Y66H), was found to have identical fluorescence spectra to those of the GFP(Y66H) protein (data not shown). Moreover, cells expressing GFPA(Y66H) were found to fluoresce 29-fold more intensely than cells expressing GFP(Y66H) (λ_{ex} = 384 nm, λ_{em} = 448 nm) when incubated at 37 °C, and 3-fold more intensely when incubated at 25 °C. Thus, previously described substitutions in the chromophore of GFP can also be used to modify the fluorescence spectra of GFPA without compromizing its improved folding characteristics

Expression of GFP5 results in dramatically improved fluorescence in mammalian tissue culture cells

We have shown that the substitutions present in the thermotolerant mutants cure the thermosensitivity of GFP expression in both bacteria and yeast, indicating that the temperature-dependent misfolding of apo-GFP may be a common phenomenon during *in vivo* expression. Therefore, the substitutions present in GFPA and GFP5 may be important for improving the performance of GFP as a marker in a wide variety of cell types, especially those that require high incubation temperatures. To examine this possibility, we compared expression of wild-type GFP and GFP5 after transient transfection of mammalian tissue culture cells. In addition, we compared the effect of the

Figure 9



Transient expression of GFP variants in Cos-7 cells. **(a)** Representative fields of cells expressing GFP, GFP(S65T), GFP5 and GFP5(S65T), all 24 h after transfection. Cells were visualized using either UV- or blue-light excitation as described in Materials and methods. The number of video frames that were integrated to form each image is indicated. Scale bar = 20 μm. **(b)** Fluorescence of cells expressing GFP variants. The fluorescence intensities of transfected cells expressing GFP, GFP(S65T), GFP5 or GFP5(S65T) 24 h following transfection were measured by flow cytometry. The increase in fluorescence of cells expressing the mutant forms of GFP relative to those expressing wild-type GFP is also shown.

S65T substitution in both the GFP and the GFP5 backgrounds. The S65T variant of GFP [10] has a single excitation peak centred at 490 nm, and has been reported to fluoresce some 6-fold more intensely than GFP when excited at this wavelength. GFP(S65T) has therefore become a widely used mutant form of GFP. In Cos-7 cells grown at 37 °C, we found that expression of GFP5 resulted in substantially more fluorescence than either GFP or GFP(S65T) (Fig. 9a). As would be predicted from its fluorescence spectra, expression of GFP5 resulted in high levels of fluorescence with either UV- or blue-light excitation. We were able to quantitate the improved fluorescence under blue-light excitation by flow cytometry (Fig. 9b), which showed that cells expressing GFP5 were on average 20-fold more fluorescent than those expressing GFP. Cells expressing GFP5(S65T) were 33-fold more fluorescent than those expressing GFP, so the effect of the S65T substitution was enhanced approximately 10-fold.

Discussion

Recently, GFP has attracted great interest as the first example of a cloned reporter protein that is intrinsically fluorescent [6]. Expression of the protein appears to be problematic in some experimental systems, however. We have shown that safe expression of high levels of GFP in transgenic *A. thaliana* requires the removal of cryptic intron signals from the cDNA and compartmentalization of the protein to the endoplasmic reticulum ([13]; J.H., K.R.S., D. Prasher and S. Hodge, unpublished observations). Furthermore, poor expression of GFP at elevated temperatures has been observed in bacteria [18], yeast [19] and mammalian cells [15–17]. The occurrence of problems such as these upon expression in heterologous systems is perhaps not surprising considering that GFP has a complex post-translational maturation and has evolved in a marine organism that lives at relatively cool temperatures. We have demonstrated that maturation of GFP in *E. coli* is sensitive to temperature and that this is due primarily to misfolding of the apoprotein at elevated temperatures. Moreover, we have isolated a mutant, GFPA, containing two amino-acid substitutions that suppress this defect in both bacteria and yeast. Neither of these substitutions is in close proximity to the chromophore of folded GFP [22,23], consistent with their primary effects being on folding rather than on the environment of the chromophore. We have manipulated the spectra of GFPA by site-directed mutagenesis to produce a thermostable folding mutant, GFP5, which can be efficiently excited using either long-wave UV or blue light and the expression of which results in significantly improved levels of fluorescence in both bacterial and mammalian cells cultured at 37 °C.

Detailed studies of the effects of mutations on protein folding and aggregation (see [27] for review) suggest a number of ways in which the substitutions present in GFPA and GFP5 might confer thermotolerance on apo-GFP. In the most simple case, the substitutions could increase the thermodynamic stability of the native apoprotein. Alternatively, the substitutions might ensure that the correct folding pathway is followed by reducing the tendency of normal folding intermediates to aggregate, or by reducing the formation of off-pathway intermediates. It is also possible that the substitutions act indirectly on the folding pathway at higher temperatures, either by increasing the rate of chromophore cyclization so as to reduce the steady state levels of immature GFP species, or by increasing the affinity of partially folded apoprotein for a cellular chaperone. Biophysical analyses of variants that are trapped as apoproteins will be required in order to distinguish between these different possibilities. However, the observation that the substitutions present in GFPA increase the T_m (melting temperature, at which 50 % of the protein is denatured) of mature GFP by 4.0 °C (K.R.S. and R.G., unpublished observations) provides an indication that they may act by increasing the thermodynamic stability of the native apoprotein. As well as enhancing proper folding, the substitutions present in the thermotolerant mutants cause the proteins to accumulate in bacterial cells to higher levels than unmodified GFP. This may reflect some susceptibility of improperly folded apo-GFP to degradation by the cellular proteolytic machinery.

We have shown that oxidation of the GFP chromophore is not temperature-sensitive by measuring the reaction rate in yeast cells at both 25 °C and 37 °C (Fig. 3). An interesting point arising from this experiment is that the time constants derived for GFP at both 25 °C and 37 °C (16.2 ± 0.3 min and 5.9 ± 0.1 min, respectively) are significantly faster than the 120 min estimated for the oxidation of GFP in bacteria [10]. This observation may reflect a difference in the physiological states of yeast and bacterial cells following anaerobic growth, or perhaps the presence of a catalysing factor in yeast cells. Nevertheless, the results suggest that oxidation of the GFP chromophore has the capacity to proceed at a much higher rate than was previously thought. In some cases, therefore, the factor limiting how quickly fluorescence can be observed following protein synthesis may be the efficiency with which apo-GFP folds and undergoes cyclization, rather than the time taken for oxidation of the chromophore. This notion is supported by our observation that fluorescence was observed much earlier in mammalian tissue culture cells expressing the thermotolerant mutants than in those expressing GFP (K.R.S. and R.S., unpublished observations).

Examination of the fluorescence spectra of GFPA revealed a shift in the amplitude of the 475 nm excitation peak relative to the amplitude of the 400 nm excitation peak. As has been suggested by others [3,9], this indicates that mutations in the carboxy-terminal region of GFP are able to modulate the spectroscopic state of the chromophore by affecting its

local environment within the protein. We took advantage of this phenomenon to engineer the fluorescence spectra of GFPA by combining a third substitution, I167T [3], with the two substitutions already present in the carboxy-terminal region. The resulting protein, GFP5, has two excitation peaks (maxima at 395 nm and 473 nm) of almost equal amplitude and is thus ideal as a multi-purpose spectral variant which can be used for applications requiring either long-wavelength UV- or blue-light excitation. We were also able to manipulate the fluorescence spectra of GFPA by introducing the Y66H chromophore substitution to give a protein, GFPA(Y66H), with identical blue-shifted fluorescence spectra to those of GFP(Y66H). The observation that maturation of both GFP5 and GFPA(Y66H) is thermotolerant demonstrates that it is possible to manipulate the fluorescence spectra of GFPA by the introduction of additional substitutions into either the carboxy-terminal or the chromophore region of the protein without deleteriously affecting its improved folding characteristics. We were therefore able to combine the S65T substitution with the substitutions present in GFP5 to substantially enhance fluorescence of the S65T variant in mammalian cells (Fig. 9). Thus, the potential exists for the rational design of a range of thermotolerant proteins with varying excitation and emission properties by combining previously described spectral mutations with the substitutions present in GFPA or GFP5.

The fact that the substitutions present in GFPA and GFP5 affect the fluorescence spectra of GFP suggests that they may affect the extinction coefficient (the molar absorptivity) and/or the quantum yields of the chromophore at the two excitation wavelengths, in addition to enhancing proper folding at elevated temperatures. However, inefficient folding of apo-GFP presents a significant practical problem for the measurement of extinction coefficients. Accurate determination of an extinction coefficient requires that the precise concentration of mature fluorescent protein in a given sample be known. The results presented here suggest that, even in a soluble cell fraction, there may be appreciable amounts of improperly folded apoprotein. This conclusion is supported by the observation that the ratio of the absorption of the chromophore to that of the aromatic amino acids of GFP purified from the soluble fraction of bacterial cells grown at 25 °C was approximately 0.4 (Fig. 6). Because this value is in excess of 1.0 for either native or acid-denatured GFP isolated directly from *Aequorea* [25], it would appear that over half of the recombinant GFP in our soluble fraction lacked a functional chromophore, thus making any attempted measurement of an extinction coefficient artificially low.

We suggest that in the absence of an assay for the concentration of chromophore in a given protein sample, care must be taken when assessing the effects of substitutions on the intrinsic fluorescence properties of recombinant forms of GFP. For example, two bright mutants (W7 and W2) of the Y66W chromophore variant of GFP have recently been described that are reported to have extinction coefficients and quantum yields comparable to those of wild-type GFP [12]. The mutants contain four and six amino-acid substitutions, respectively, which are thought to act by improving the fit of the substituted tryptophan residue into the internal cavity normally occupied by tyrosine. However, the presence in both mutants of the V163A substitution, shown here to be important for improving the folding of GFP, suggests that their increased brightness may be due, at least in part, to improved folding rather than improvements in their extinction coefficients or quantum yields. Indeed, V163A may be a substitution central to improvement of the folding of GFP and derivatives, a conclusion supported by its presence amongst three substitutions in another bright mutant of GFP (C3) that is more soluble during expression in *E. coli* [28].

Conclusions

We have shown that the substitutions present in GFPA and GFP5 suppress a temperature-sensitive defect in the folding of apo-GFP in *E. coli*. As these substitutions also suppress the thermosensitivity of GFP in yeast cells and significantly improve levels of fluorescence in mammalian cells cultured at 37 °C, as well as in *Drosophila melanogaster* embryos incubated at 25 °C (A. Brand, personal communication), it would appear that this folding defect is a common phenomenon during GFP expression *in vivo*. In addition to coding for improved folding and spectral characteristics, the modified *gfp* genes described here have an altered codon usage necessary to ensure proper mRNA processing in plants ([13]; J.H., K.R.S., D. Prasher and S. Hodge, unpublished observations), and which may be advantageous for expression in other heterologous cell types. Indeed, 'humanization' of the *gfp* coding sequence has been reported to aid in the expression of GFP in mammalian tissue culture cells [29,30]. Thus, we expect that the thermotolerant folding mutants described in this work, and spectral variants derived from them, should be of significant benefit for GFP-based applications in many experimental systems, in particular those, such as mammalian cells, that use relatively high incubation temperatures.

Materials and methods

Mutagenesis and plasmid construction

The sequence of *mgfp4* was mutated by PCR in the presence of limiting nucleotide concentrations. The template plasmid was pBS*mgfp4* ([13]; J.H., K.R.S., D. Prasher and S. Hodge, unpublished observations), and the primers were the T3 and T7 primers (New England Biolabs) that are complementary to the flanking T3 and T7 promoters present in the vector sequence. Four separate reactions (30 cycles of 30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C using Taq DNA Polymerase from Promega) were carried out, each with the concentration of a different nucleotide reduced from 200 μM to 20 μM. The amplified fragments were pooled, cleaved with *Kpn*I and *Eco*RI and cloned downstream of the *lac* promoter of pBluescript II KS (+) (Stratagene). The mutant library was transformed into *E. coli* strain XL1-Blue (Stratagene) and incubated overnight at 37 °C on

TYE agar containing 50 μg ml^{-1} ampicillin and 1 mM IPTG. Colonies were illuminated with a long-wavelength UV lamp (UVP Model B 100 AP) and visually screened for increased fluorescence. The coding regions of two mutant genes (*mgfpA* and *mgfpB*) as well as that of *mgfp4* were amplified by PCR (30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C using VENT DNA Polymerase from New England Biolabs) using the optGFP5 oligo 5′-GGC**GGATCC**AAG-GAGATATA**ACA**AATG AGT AAA GGA GAA GAA CTT TTC ACT-3′ (*Bam*HI site underlined, 'phage Shine–Dalgarno sequence in italics and plant translation initiation context sequence in bold) as the forward primer and the oligo 5′GGC**GAGCTC**T**TA** TTT GTA TAG TTC ATC CAT GCC-3′ (*Sac*I site underlined and GFP stop codon in bold) as the reverse primer. The amplified fragments were cleaved with *Bam*HI and *Sac*I and cloned downstream of the *lac* promoter of pUC119 [31]. The mutations responsible for the bright phenotypes of *mgfpA* and *mgfpB* were then located by recombination of the mutant genes with *mgfp4*. The pUC119 derivatives containing *mgfpA* and *mgfpB* were cleaved with either *Bam*HI and *Nco*I, *Nco*I and *Cla*I, or *Cla*I and *Sac*I. The restriction fragments were gel-purified and ligated to the *mgfp4* pUC119 derivative that had been cleaved with the same combination of enzymes and gel-purified. These and the parent constructs were introduced into XL1-Blue cells and incubated overnight at 37 °C on agar plates containing ampicillin and IPTG. Comparison of the fluorescence of colonies containing the various constructs revealed that the mutation(s) responsible for the bright phenotypes of both *mgfpA* and *mgfpB* were contained within the 336 base-pair *Cla*I–*Sac*I fragment at the 3′ end of the gene. These fragments were cloned into the phage vector M13mp19 (New England Biolabs) and sequenced from the Universal primer using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corporation).

Site-directed mutagenesis to produce the *mgfp5* and *mgfp(Y66H)* genes was by PCR amplification of *mgfp4* using appropriate mutagenic primers. Mutant fragments were subsequently inserted in place of the corresponding non-mutant fragments of *mgfp4*; *mgfpA(Y66H)* and *mgfp5(S65T)* were constructed in the same way except that the *mgfpA* and *mgfp5* genes, respectively, were used as the templates for PCR amplification with mutagenic primers.

For bacterial expression, *Bam*HI–*Sac*I fragments containing the *mgfp4*, *mgfpA*, *mgfp5*, *mgfp(Y66H)* and *mgfpA(Y66H)* genes were excised from the pUC119 derivatives and cloned downstream of the IPTG-inducible *trc* promoter of the expression vector pSE380 (Invitrogen), to give the plasmids pSE-GFP, pSE-GFPA, pSE-GFP5, pSE-GFP(Y66H) and pSE-GFPA(Y66H), respectively. Expression from the *trc* promoter of pSE380 is tightly regulated as a result of the presence on the plasmid of the *lacI*^{q} gene. For yeast expression, *Bam*HI–*Sac*I fragments containing the *mgfp4* and *mgfpA* genes were inserted downstream of the constitutive *ADH1* promoter of pVT-103-U [32], a yeast multicopy episomal plasmid containing the *URA3* selectable marker. The resulting plasmids were pVT-GFP and pVTGFPA, respectively. For expression in mammalian tissue culture cells, *gfp* [5], *gfp(S65T)* [10], *mgfp5* and *mgfp5(S65T)* were amplified by PCR using optGFP5 as the forward primer and the oligo 5′-GGC**AGATCT** TTA TTT GTA TAG TTC ATC CAT GCC-3′ (*Bgl*II site underlined) as the reverse primer. The amplified fragments were cleaved with *Bam*HI and *Bgl*II and cloned into the *Bam*HI site that lies downstream of the CMV early promoter of the pcDNA3 expression vector (Invitrogen). The resulting vectors were named pcGFP, pcGFP(S65T), pcGFP5 and pcGFP5(S65T).

Polyhistidine-tagging was achieved by the addition of six histidine codons to the 3′ ends of the modified *gfp* genes by PCR. The genes were amplified using optGFP5 as the forward primer and the oligo 5′-GCC-**GAGCTC** TTA **GTG GTG GTG GTG GTG GTG** TTT GTA TAG TTC ATC CAT GCC-3′ (*Sac*I site underlined, histidine codons in bold) as the reverse primer. The amplified fragments were cleaved with *Bam*HI and *Sac*I and cloned downstream of the *trc* promoter of pSE380 to give the expression plasmids pSE-GFPHis, pSE-GFPAHis and pSEGFP5His.

Determination of chromophore oxidation rates

Strains of *S. cerevisiae* MGLD-4a (*MATa, leu2, ura3, his3, trp1, lys2*) containing either pVT-GFP or pVT-GFPA were incubated anaerobically (Becton-Dickinson BBL GasPak Pouch) overnight at 30 °C in synthetic drop-out media lacking uracil [33]. Following admission of air to the pouch, 1 ml of each culture was immediately centrifuged for 1 min at 13 000 rpm and resuspended in 0.5 ml aerated and prewarmed PBS (pH 7.4) containing 8 mM NaN_3 as a metabolic inhibitor. Cell suspensions were placed immediately into pre-warmed cuvettes held within the fluorimeter carousel and the time course of fluorescence ($\lambda_{ex} = 397$ nm, $\lambda_{em} = 508$ nm) development measured.

Preparation of soluble and insoluble cell fractions

Cells were grown in 1.5 ml of 2xTY broth to an absorbance of 0.2 at 600 nm and protein expression induced overnight with 0.2 mM IPTG. The cultures were centrifuged at 13 000 rpm for 2 min, resuspended in 500 μl 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 100 μg ml^{-1} lysozyme, 0.1 % Triton X-100 and incubated at 30 °C for 15 min. Cells were then lysed by sonication (5 × 15 sec) using a Heat Systems (Model CL4) sonicator and centrifuged at 13 000 rpm for 15 min at 4 °C. The supernatant (soluble fraction) was removed and stored at −70 °C until used. The pellet (insoluble fraction) was washed once with 500 μl 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5 % Triton X-100, resolubilized for 1 h at room temperature in 500 μl resolubilization buffer (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCl, pH 6.3) and stored at −70 °C until used.

Western-blot analysis

Total protein from *E. coli* cells (diluted to an optical density of 0.2 at 600 nm) was prepared by boiling cells in an equal volume of 2x SDS sample buffer for 3 min. SDS–PAGE using 10 % gels and western blots were carried out according to standard protocols [34]. Primary antibodies were polyclonal rabbit anti-GFP (generous gift of S. Santa-Cruz) used at a dilution of 1/2 000. Antibodies were detected with iodinated Protein A (Amersham) and bands visualized and quantified using a Molecular Dynamics Phosphorimager.

Protein purification

For the purification of polyhistidine-tagged GFP for absorption measurements, soluble and insoluble fractions of cells containing pSE-GFPHis grown at 25 °C and 37 °C, respectively, were prepared as described above. GFP was purified from the fractions on Ni-chelate columns using the Ni–NTA Spin Kit (Qiagen). Purification from the soluble fraction was carried out according to the manufacturer's protocol for the purification of polyhistidine-tagged proteins under native conditions. After clearance of cellular debris from the insoluble fraction by centrifugation at 13 000 r.p.m. for 30 min, purification was carried out according to the protocol for purification of polyhistidine-tagged proteins under denaturing conditions, except that the protein was eluted with resolubilization buffer (see above) containing 250 mM imidazole.

For the purification of native polyhistidine-tagged proteins for fluorescence spectroscopy, cells were grown in 100 ml of 2xTY broth at 37 °C to an absorbance of 0.2 at 600 nm and then induced overnight with 0.5 mM IPTG. Cells were harvested by centrifugation at 6 000 r.p.m. for 10 min and lysed by resuspension in 4 ml 20 mM Tris–HCl (pH 7.9), 500 mM NaCl, 5 mM imidazole, 0.1 % sarkosyl, 0.1 % deoxycholate, 2.25 M guanidine–HCl. Nucleic acids were precipitated by the addition of 5 ml isopropanol and removed by centrifugation at 10 000 rpm for 10 min. Following 0.45 μm filtration, fluorescent polyhistidine-tagged proteins were purified from the supernatant on Ni-chelate columns (Qiagen) and eluted with 2 ml 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 150 mM imidazole. For all purifications, protein purity was assayed by SDS–PAGE and found to be greater than 95 %. Protein concentrations were determined by Bradford assay (Bio-Rad Protein Assay kit) using bovine serum albumin as a standard.

Spectroscopy

Absorbance spectra were recorded on a Cary 3 UV-Visible Spectrophotometer (Varian) at 25 °C. The optical path-length was 1 cm.

Fluorescence measurements were made on a Hitachi F-4500 fluorimeter using 4mm/10mm cuvettes. The bandpass for both the excitation and the emission monochromators was 5 nm, the scan speed 240 nm min^{-1} and the response time automatically adapted by the device. All fluorescence spectra were recorded at 25 °C and were corrected following the supplier's procedure for calibration of the fluorimeter using Rhodamine-B as standard. Emission spectra were recorded at a fixed wavelength of the excitation maximum, and excitation spectra at a fixed wavelength of the emission maximum.

Transient transfection and flow cytometric analysis of mammalian tissue culture cells

Cos-7 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 % fetal calf serum, and transfected by electroporation [35] using 20 µg plasmid DNA per 10^6 cells. Transiently transfected cells were grown on Lab-Tek Chambered Coverglass (Nunc) for 24 h at 37 °C prior to fluorescence microscopy. For flow cytometric analysis, cells were grown in 100 mm dishes for 24 h following transfection, trypsinized, and resuspended in PBS. The fluorescence intensities of the transfected cells were immediately analysed by flow cytometric analysis using a FACScan analyser and LYSYS II software (Beckton Dickinson). Samples were excited with an argon ion laser and fluorescence was detected through the FLI filter (530DF30). The mean fluorescence intensity for each sample was calculated by averaging the values for the first 10 000 cells measured using detector settings calibrated to ignore the low intensity autofluorescence of non-transfected cells.

Fluorescence microscopy

Fluorescent yeast colonies and Cos-7 cells were visualized with an inverted fluorescence microscope (Leitz DM-IL) fitted with filter sets suitable for UV- (Leitz-D; excitation filter 355–425 nm, dichromatic mirror 455 nm, suppression filter 460 nm) or blue- (Leitz-I3; excitation filter 450–490 nm, dichromatic mirror 510 nm, suppression filter 520 nm) light excitation of GFP. Epifluorescence images were recorded using a Sony 3-chip CCD video camera and F100-MPU integrating frame store, connected to a NuVista+ video digitizer in an Apple Macintosh Quadra 800 computer.

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